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The principal goal of the proposed studies is to determine if 1,25-dihydroxy-vitamin D3 and some of its analogues inhibit proliferation of human breast cancer cells by altering the expression and function of endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) for lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P). Estrogen receptor positive and estrogen receptor negative cells express predominantly Edg-2 and Edg-4 Rs for LPA and Edg-3 for S1P, which transduce proliferative responses by direct nuclear signaling, through MAP kinase, and stimulate secretion of type II insulin-like growth factor (IGF-II). The proliferation of breast cancer cells induced by LPA and S1P is inhibited significantly by 10-10-10-8 M 1,25-dihydroxy-vitamin D3 analogue. We aim to delineate the effects of 1,25-dihydroxy-vitamin D3 analogues on Edg R expression and biochemical signaling, IGF-II receptor expression and secretion of IGF-II. Results are expected to further our understanding of abnormal growth regulatory mechanisms in breast cancer and may lead to Edg R directed modalities of treatment.

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Hana Polin 12/26/97

PI - Signature Date

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(5) **INTRODUCTION**:

The original research plan was designed to investigate the possibility suggested by preliminary data that 1,25-dihydroxy-vitamin D3 (1,25-diOH-D3) and several of its analogues enhance proliferation of estrogen receptor-positive breast cancer cells in culture by stimulating secretion of type II insulin-like growth factor (IGF-II) and upregulating expression of the type I receptor for IGFs (IGFR1), whereas 1,25-diOH-D3 suppresses proliferation of estrogen receptor-negative breast cancer cells. Extensive studies conducted by Dr. Dolezalova in the past 10 months have not shown reproducible effects of 1,25-diOH-D3 on either spontaneous secretion of IGF-II by estrogen receptor-positive breast cancer cells or proliferative responses of the same estrogen receptor-positive breast cancer cells to exogenous synthetic IGF-II. Investigations thus were re-directed to effects of 1,25-diOH-D3 on proliferation of human breast cancer cells evoked by a new family of lysolipid phosphate (LLP) growth factors, which stimulate proliferation by direct nuclear signaling and by stimulation of production of IGF-II by the breast cancer cells. Dr. Dolezalova has recently demonstrated prominent expression of G protein-coupled receptors (GPCRs) for LLPs by both estrogen receptor-positive and –negative breast cancer cells, stimulation of proliferation of breast cancer cells by LLPs, augmentation of breast cancer cell production of IGF-II by LLPs, and very striking suppression of LLP-elicited proliferation of breast cancer cells by 1,25-diOH-D3. The major goal for the next period of research is to elucidate the mechanisms of 1,25-diOH-D3 suppression of breast cancer cell proliferative and biochemical responses to the LLPs.

(6) **BODY**:

a) The first task of the period was to attempt to reproduce preliminary data from Dr. Cheryl D. Love-Schimenti, the first fellow in this program. However, the results obtained did not confirm Dr. Love-Schimenti's initial observations nor reveal significant enhancement by 1,25-diOH-D3 of any biochemical or cellular activity of estrogen receptor-positive human breast cancer cells. Proliferative responses of breast cancer cells were quantified by microscopic counts and by reports of activation of promoters of immediate-early growth-related genes from response element-luciferase constructs transfected into the breast cancer cells prior to stimulation. In three consecutive studies of the MCF-7 line of human estrogen-receptor positive breast cancer cells, which Dr. Love-Schimenti had used in preliminary investigations, 10^{-11} M and 10^{-8} M 1,25-diOH-D3 changed the spontaneous level of proliferation to respective means +/- S.D. of 143% +/- 52% and 140% +/- 78% of the control, which both lacked statistical significance. In the same experiments, 10^{-10} M estradiol increased proliferation of MCF-7 cells by a mean +/- S.D. of 295% +/- 162%, that was reduced insignificantly by means of only 17% and 23% by 10^{-11} M and 10^{-8} M 1,25-diOH-D3. Production of IGF-II by MCF-7 cells, as assessed by radioimmunoassay, attained a range of 1.4 to 3.7 ng/ml after 24 hr, which was not altered significantly by 1,25-diOH-D3.
Conclusions: The active vitamin D metabolite 1,25-diOH-D3 did not significantly

modify spontaneous or estradiol-stimulated proliferation of estrogen receptor-positive MCF-7 breast cancer cells, nor their level of secretion of IGF-II.

b) Characterization of Expression of LLP Receptors and Responses to LLPs of Human Cultured Breast Cancer Cells-

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are LLPs produced by platelets, macrophages, other leukocytes, epithelial cells and some tumor cells, which affect normal cells by stimulating proliferation, increasing survival, suppressing apoptosis and initiating or enhancing diverse cytoskeleton-based functions. Endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) Edg-1, -3 and –5 bind S1P and Edg-2, -4, and –7 bind LPA with high-affinity resulting in transduction of ras- and rho-dependent proliferation-stimulating signals to the serum response element (SRE) of promoters of numerous immediate-early growth-related genes and rho-dependent signals to cellular adhesion, migration and secretion. In collaboration with her mentor, Dr. Edward J. Goetzl, Dr. Dolezalova has examined the Edg Rs and responses to LPA and S1P of the MCF-7 and estrogen receptor-negative MDA-MB-453 human breast cancer cells.

MCF-7 and MDA-MB-453 both expressed Edg-2, -3, -4 and –5, but not Edg-1, as determined by semiquantitative RT-PCR and Western blots with monoclonal antibodies to substituent peptides of each Edg R. The levels of Edg-3 in both lines were similar and higher than previously studied human cells, those of Edg-2 also were similar but much lower than Edg-3, and Edg-4 and –5 were higher in the MCF-7 than MDA-MB-453 cells. The proliferation of both breast cancer cell lines was stimulated significantly by 10^{-8} M to 10^{-6} M LPA and S1P, as assessed by cell counts, and by 10^{-10} M to 10^{-6} M LPA and S1P, as quantified by reports of SRE-luciferase plasmids introduced by transfection which were standardized by the levels of concurrent reports of Renilla luciferase constructs. The SRE-luciferase luminometric activity in MCF-7 cells was increased by respective mean maxima of 37- and 85-fold with LPA and S1P and in MDA-MB-453 cells by 24- and 26-fold. The secretion of radioimmunoreactive IGF-II by MCF-7 cells was increased significantly by means of up to 3.2-fold by LPA and 5.5-fold by S1P. The secretion of IGF-II by MDA-MB-453 cells also was augmented by LLPs, but the extent of the increment could not be calculated because the baseline levels were below that detectable by the radioimmunoassay. The increases in IGF-II secretion were significant for tumor cell proliferation, as addition of synthetic IGF-II to attain an equivalent increment stimulated SRE-luciferase reports in MCF-7 cells by 200% to 300% of control values. Further, neutralizing anti-IGF-II monoclonal antibody and anti-IGFR1 monoclonal antibody, which blocks binding of IGFs to the IGFR1, both suppressed proliferative responses of MCF-7 cells to optimal concentrations of LPA and S1P by up to 30% to 60%. These data suggested that human breast cancer cells respond to LPA and S1P through Edg Rs by dual

mechanisms of direct nuclear signaling and enhancement of endogenous secretion of IGF-II.

Conclusions: Estrogen receptor-positive and –negative breast cancer cells express high levels of Edg Rs for LLPs and respond with enhanced proliferation to both LPA and S1P, by direct and IGF-II-mediated mechanisms. This system may allow more productive investigations of the effects of vitamin D on breast cancer.

c) **Effects of 1,25-diOH-D3 on Breast Cancer Cell Proliferation Evoked by LLPs-**

The results of recent studies have shown that 1,25-diOH-D3 suppresses proliferative responses of human breast cancer cells to LPA and S1P. The SRE-luciferase reports from MCF-7 cells were increased by a mean of 9.3-fold (n=6) by 10^{-7} M S1P and those from MDA-MB-453 cells were increased by a mean of 13.4-fold (n=3). At a concentration of 10^{-8} M, 1,25-diOH-D3 suppressed the response of MDA-MB-453 cells to 10^{-7} M S1P by a mean of 91%.

Conclusions: The exuberant proliferative and protein synthetic responses of human breast cancer cells in culture to both LPA and S1P, the involvement of IGF-II in the effects of the LLPs, and the striking inhibitory effects of 1,25-diOH-D3 on these responses, will allow systematic studies of the mechanisms by which vitamin D suppresses growth of both estrogen receptor-negative and –positive breast cancer.

d) **Revised Research Plan for the Term of Grant Award-**

To define the mechanisms of effects of vitamin D on breast cancer cells, studies will be designed to accomplish the following **specific aims:**

i. Delineate the conditions under which 1,25-diOH-D3 maximally inhibits the proliferation of LPA- and S1P-stimulated MCF-7 and MDA-MB-453 breast cancer cells, as assessed by cell counts, ^{3}H-thymidine uptake, and SRE-luciferase reports.

ii. Quantify the effects of maximally-inhibitory exposure times and concentrations of 1,25-diOH-D3 on expression of Edg Rs by breast cancer cells.

iii. Determine effects of maximally-inhibitory exposure times and concentrations of 1,25-diOH-D3 on increases in $[Ca^{++}]i$ evoked by LPA and S1P in breast cancer cells.

iv. Elucidate alterations in LPA- and S1P-stimulated breast cancer cell cycle regulation by maximally-inhibitory exposure times and concentrations of 1,25-diOH-D3, initially by flow cytometric analyses of BrdU-labeled cells and Western blot quantification of cyclin-cdk inhibitor p21/Waf1.

v. Assess effects of maximally-inhibitory exposure times and concentrations of 1,25-diOH-D3 on secretion of IGF-II and expression of IGFR1 by LPA- and S1P-stimulated breast cancer cells, and on their responsiveness to synthetic IGF-II.

(7) Appendices

1) List of Key Research Accomplishments:

+ Characterization of the profile of Edg Rs for LPA and S1P expressed by MCF-7 (estrogen receptor-positive) and MDA-MB-453 (estrogen receptor-negative) human breast cancer cells in culture.

+ Demonstration of the stimulation of proliferation of both lines of breast cancer cells by LPA and S1P.

+ Development and transfection into breast cancer cells of SRE-luciferase and related reporters of nuclear signaling of immediate-early growth-related genes by LPA and S1P.

+ Demonstration of stimulation of SRE-luciferase reports from breast cancer cells by LPA and S1P.

+ Discovery of enhancement by LPA and S1P of production and secretion of IGF-II by breast cancer cells, and of the major contribution of endogenous IGF-II to proliferative and SRE-luciferase responses.

+ Finding that 1,25-diOH-D3 suppresses LPA- and S1P-induced proliferation and nuclear signaling significantly by up to 90%, without altering unstimulated responses.

2) Reportable Outcomes

Full Publications-

Goetzl, E.J., **Dolezalova, H.,** Kong, Y., and Zeng, L. (1999) Dual Mechanisms for Lysophospholipid Induction of Proliferation of Human Breast Carcinoma Cells. Cancer Research **59:** 4732-4737.

Abstract

Goetzl, E.J., Dolezalova,H., Kong,Y., Zeng,L. (1999) Dual Mechanisms for Endothelial Differentiation Gene-encoded Receptor (Edg R) Mediation of Proliferation of Human Breast Cancer Cells. FASEB J. 13: A136

[CANCER RESEARCH 59, 4732–4737, September 15, 1999]

Dual Mechanisms for Lysophospholipid Induction of Proliferation of Human Breast Carcinoma Cells[1]

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ABSTRACT

Endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) Edg-1, Edg-3, and Edg-5 bind sphingosine 1-phosphate (S1P), and Edg-2 and Edg-4 Rs bind lysophosphatidic acid (LPA). LPA and S1P initiate ras- and rho-dependent signaling of cellular growth. Cultured lines of human breast cancer cells (BCCs) express Edg-3 > Edg-4 > Edg-5 > or = Edg-2, without detectable Edg-1, by both assessment of mRNA and Western blots with rabbit and monoclonal mouse anti-Edg R antibodies. BCC proliferation was stimulated significantly by 10^{-9} M to 10^{-6} M LPA and S1P. Luciferase constructs containing the serum response element (SRE) of growth-related gene promoters reported mean activation of BCCs by LPA and S1P of up to 85-fold. LPA and S1P stimulated BCC secretion of type II insulin-like growth factor (IGF-II) by 2–7-fold, to levels at which exogenous IGF-II stimulated increased proliferation and SRE activation of BCCs. All BCC responses to LPA and S1P were suppressed similarly by pertussis toxin, mitogen-activated protein kinase kinase inhibitors, and C3 exoenzyme inactivation of rho, suggesting mediation by Edg Rs. Monoclonal anti-IGF-II and anti-IGFR1 antibodies suppressed proliferation and SRE reports of BCCs to LPA and S1P by means of up to 65%. Edg Rs thus transduce LPA and S1P enhancement of BCC growth, both directly through SRE and indirectly by enhancing the contribution of IGF-II.

INTRODUCTION

The lysolipid phosphate mediators LPA[3] and S1P are generated enzymatically from membrane lipid precursors of many different types of normal and malignant cells (1, 2). Extracellular LPA and S1P both stimulate cellular proliferation, differentiation, survival, adhesion, aggregation, and other specific functions (3–5). A recently characterized subfamily of at least five G protein-coupled receptors, which are encoded by *edg*s, bind and transduce signals from LPA or S1P (6–10). Two homology clusters with greater structural similarity and shared ligand specificity are composed of the *edg*-encoded G protein-coupled receptors (Edg Rs) Edg-1, Edg-3, and Edg-5 set of S1P Rs and Edg-2 and Edg-4 LPA Rs. The capacity of LPA and S1P to improve cellular survival is in part a result of suppression of apoptosis by several distinct mechanisms (11, 12). LPA and S1P stimulate cellular proliferation directly by eliciting the serum response factor and ternary complex factor transcription factors, which together bind to and activate the SRE in promoters of many immediate-early genes (13). The involvement of SRE-dependent mechanisms in mediating LPA and S1P enhancement of proliferation has not been examined carefully in malignant cells, nor has the possibility of effects of LPA and/or S1P on polypeptide growth factors necessary for optimal tumor growth.

Functional Edg receptors and proliferative responses to LPA and S1P thus were characterized in the ER-positive MCF-7 cultured line of human BCCs and the MDA-MB-453 ER-negative line of BCCs. The relative contributions of direct SRE-dependent induction of transcription and of enhancement of production of IGF-II in proliferative responses to LPA and S1P also were determined in these BCCs.

MATERIALS AND METHODS

Chemical Reagents and Antibodies. The sources of chemicals were: S1P and sphingosine (Biomol, Plymouth Meeting, PA); LPA, phosphatidic acid, 1-β-D-galactosyl-sphingosine (psychosine), and fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO); and human IGF-II (Peprotech, Inc., Rocky Hill, NJ). Cells were treated with PTX (Calbiochem, Inc., La Jolla, CA), recombinant *Clostridium botulinum* C3 ADP-ribotransferase (C3 exoenzyme; List Biological Laboratories, Inc., Campbell, CA), which ADP-ribosylates rho specifically, and the MEK inhibitor 2′-amino-3′-methoxyflavone (PD98059; Calbiochem) as described (10, 14). Mouse monoclonal antibodies specific for substituent peptides of human Edg-3 (amino acids 1–21), Edg-4 (amino acids 9–27), and Edg-5 (amino acids 303–322) have been described (12, 15), the immunogens for which were selected from sequences of high homology among humans and rodents. The expected cross-reaction with corresponding rodent Edg Rs has been confirmed by the identical recognition of human and rat Edg-5 Rs. The cross-reactivity of each antibody with heterologous Edg proteins was <1%, as determined by Western blots of 0.1–100 μg of membrane proteins isolated from HTC4 rat hepatoma cells stably transfected with human Edg-2, Edg-3, Edg-4, or Edg-5 (12, 15). Each monoclonal IgG was purified by protein A affinity-chromatography (Pierce Chemical Co.) and used to develop Western blots at 0.1–0.3 μg/ml (15). A mouse monoclonal IgG1 that specifically neutralizes activity of human/rat IGF-II, but not IGF-I (Upstate Biotechnology, Inc., Lake Placid, NY), and a mouse monoclonal antibody, termed α-IR3, which blocks binding of IGF-II to IGFR1 (Oncogene Science, Cambridge, MA), were purchased. A rabbit polyclonal antiserum to rodent and human Edg-2 was kindly provided by Dr. Jerold Chun (University of California–San Diego, San Diego, CA).

Cell Culture and Quantification of Cellular Proliferation. Layers of ER-positive MCF-7 (ATCC # HTB-22) and ER-negative MDA-MB-453 (ATCC# HTB-131) human BCCs were cultured in DMEM with 4.5 g/100 ml of glucose, 10% FBS, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin (complete DMEM) to 100% confluence and relayered every 3–4 days to 25–30% confluence. To assess proliferation, replicate layers of 1×10^4 BCCs were cultured in 48-well plates in complete DMEM for 4 h, washed once, and cultured for 20 h in serum-free DMEM. Some wells were pretreated with PTX for 6 h, C3 exoenzyme for 30 h, or MEK inhibitor for 2 h. Antisera were added, followed in 1 h by lipid stimuli and incubation for 72 h. Then wells were washed two times with Ca^{2+}- and Mg^{2+}-free Hanks' solution, and the cells were harvested in 0.2 ml of EDTA-trypsin solution for staining with trypan blue and eosin and quantification by microscopic counting of 10 1-mm^3 fields in a hemocytometer.

Reverse Transcription-PCR Analysis of Edg Rs. Total cellular RNA was extracted by the TRIzol method (Life Technologies, Inc., Grand Island, NY), from suspensions of BCCs and lines of stably transfected rat HTC4 hepatoma cells, that all had low background expression of native Edg Rs, and each overexpressed one recombinant human Edg R. A Superscript kit (Life Technologies, Inc.) was used for reverse transcription synthesis of cDNAs. PCR began with a "hot start" at 94°C for 3 min; Taq DNA polymerase was added, and amplification was carried out with 35 cycles of 30 s at 94°C, 2 min at 55°C, and 1 min at 72°C. Two μCi of [α-^{32}P]dCTP were added to some sets

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[3] The abbreviations used are: LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; *Edg*, endothelial differentiation gene; SRE, serum response element; ER, estrogen receptor; BCC, breast cancer cell; MEK, mitogen-activated protein kinase kinase; PTX, pertussis toxin; IGF-II, type II insulin-like growth factor; IGFR, IGF receptor; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

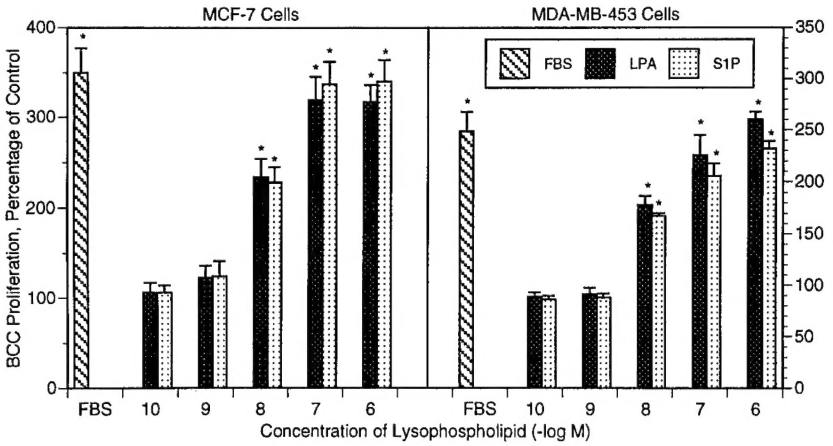

Fig. 3. Stimulation of proliferation of MCF-7 and MDA-MB-453 BCCs by LPA and S1P. *Columns,* means of the results of three studies performed in duplicate; *bars,* SD. FBS is the 2% FBS-positive control. The serum-free medium alone controls (100%) were 1.5, 1.4, and 1.5 × 10^4/well in the three studies of MCF-7 BCC proliferation and 1.1, 1.2, and 1.1 × 10^4/well in the three studies of MDA-MB-453 BCC proliferation. The levels of significance of increases above medium control proliferation were determined by a paired Student *t* test; *, $P < 0.01$.

different amounts of first-strand cDNAs prepared from MCF-7 and MDA-MB-453 BCCs were amplified initially to allow selection of a volume of each that provided equally intense cDNA bands for the internal standard G3PDH. With this standard approach, the mRNA from both human BCC lines was found to encode similarly high levels of Edg-3 R but had no detectable Edg-1 R message (Fig. 1). The ER-negative MDA-MB-453 BCCs had higher levels of mRNA encoding the Edg-2 R, whereas the ER-positive MCF-7 BCCs had higher levels of mRNA for Edg-4 and Edg-5.

RadioPCR has been used to assess levels of mRNA specific for other G protein-coupled Rs, but not Edg Rs (16). Thus, an initial study examined mRNA from four lines of rat HTC4 hepatoma cells, which were stably transfected with individual human Edg Rs 2 to 5 (Table 1). The rank order of levels of mRNA for endogenous Edg Rs in HTC4 cells prior to transfection was Edg-2 ≫ Edg-3 > Edg-4 > Edg-5, without detectable Edg-1 mRNA. The level of mRNA for the transfected Edg R in each line was much higher than background (Table 1). In this frame of reference, the levels of BCC mRNA encoding Edg-3 were nearly as high as the index transfectant and > Edg-4 > Edg-5 ≥ Edg-2, without any Edg-1 mRNA. The differences in relative amounts of mRNA for each Edg R between the two lines of BCCs were the same as for standard PCR (Fig. 1).

Western blots developed with polyclonal anti-Edg-2 R and monoclonal anti-Edg-3, anti-Edg-4, and anti-Edg-5 antibodies showed one predominant protein of expected size in extracts of each of the four lines of HTC4 cell transfectants (Fig. 2). Electrophoresis of over three times more protein from untransfected control HTC4 cells than transfectants did not show Edg-3, Edg-4, or Edg-5 protein antigen, but a faint band of Edg-2 protein was detected that might reflect the higher endogenous levels of mRNA encoding this R (Table 1). The results of BCC Western blots confirmed expression of Edg proteins representing both LPA R and S1P R subtypes, with a predominance of Edg-3 R in both BCC lines (Fig. 2). In contrast to expectations from PCR results, however, MCF-7 BCCs had higher levels of Edg-2 as well as Edg-4 and Edg-5 proteins than MDA-MB-453 BCCs. The Edg-4 R protein of both BCC lines was consistently M_r 2000–3000 smaller than the recombinant human Edg-4 R protein, but the basis for the difference has not yet been elucidated.

Functional and Biochemical Responses of BCCs to LPA and S1P. The proliferation of both lines of BCCs was assessed by counting viable cells after 72 h (Fig. 3). Proliferation of MCF-7 BCCs was increased significantly by 10^{-8} M to 10^{-6} M LPA and S1P to maximum levels similar to those attained by 2% FBS. In parallel studies of MDA-MB-453 BCCs, proliferative responses to LPA and S1P were similar to those of MCF-7 BCCs, with significant increases evoked by 10^{-8} M to 10^{-6} M LPA and S1P (Fig. 3).

Activation of SRE in the promoters of diverse growth-related genes is a fundamental characteristic of the growth-promoting potential of LPA and S1P. BCCs thus were transfected with an SRE-firefly luciferase construct and 1/20 the amount of a Renilla luciferase-CMV construct as an internal standard for consistency of transfection. LPA and S1P increased the mean levels of standardized luciferase luminometric activity in ligand concentration-dependent relationships by maxima of up to 37-fold and 85-fold, respectively, in MCF-7 BCCs (Fig. 4). Similar responses to the same concentrations of LPA and S1P

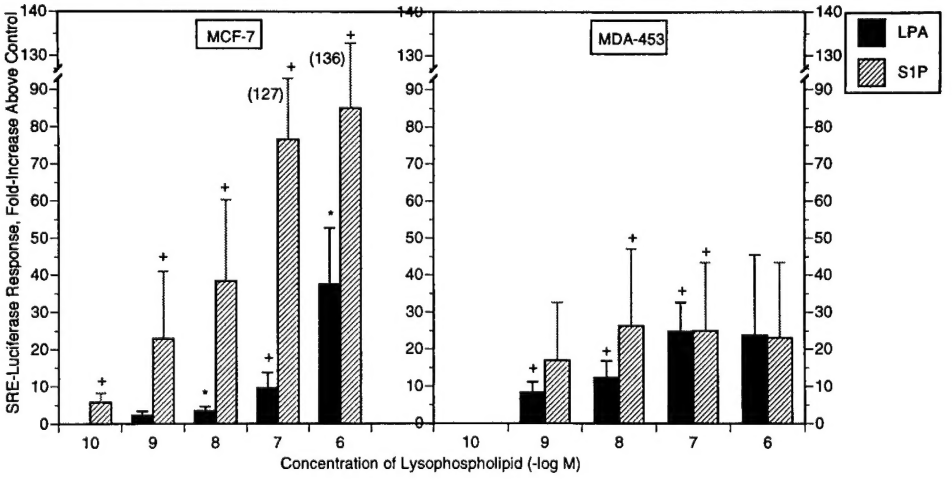

Fig. 4. SRE reporter assay of LPA and S1P stimulation of human BCCs. *Columns,* means of the results of three studies performed in duplicate; *bars,* SD. The medium alone control values were 1272, 957, and 352 luminometer units for MCF-7 BCCs and 269, 715, and 1401 for MDA-MB-453 BCCs. The statistical methods and symbols are the same as in Fig. 3, except that + = $P < 0.05$.

Table 2 *Pharmacological inhibition of LPA and S1P signaling to the SRE-Luciferase reporter in BCCs*

Each number is the mean of results of two studies performed in duplicate and presented as the percentage of inhibition of the control responses to 10^{-7} M LPA and 10^{-7} M S1P in serum-free DMEM without inhibitors (0% inhibition). Inhibitor conditions were 50 ng/ml of PTX for 6 h, 5 μM MEK inhibitor (MEK INH) for 1 h, and 10 μg/ml of C3 exoenzyme for 30 h.

	MCF-7 BCCs			MDA-MB-453 BCCs		
	PTX	MEK INH	C3 exoenzyme	PTX	MEK INH	C3 exoenzyme
LPA	74	41	41	80	69	75
S1P	60	37	44	78	61	79

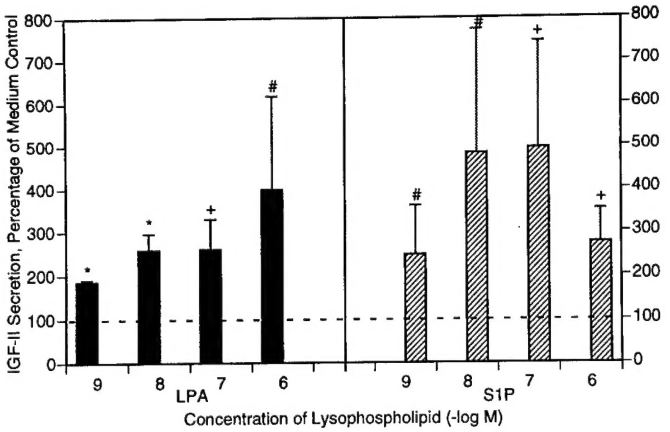

Fig. 5. Stimulation by LPA and S1P of MCF-7 BCC secretion of IGF-II. *Columns*, means of the results of three studies; *bars*, SD. Secretion of IGF-II in medium alone was 1.7, 3.0, and 4.1 ng/ml in the three studies. The statistical methods and symbols are the same as in Fig. 4, except that # = $P = 0.05$.

were detected in MDA-MB-453 BCCs, where the respective mean maxima were 24-fold and 26-fold.

Pharmacological inhibitors known to suppress one or more components of the pathways by which Edg Rs signal nuclear events were applied in BCCs transfected with the SRE-luciferase reporter. Suppression of Gi protein activity by PTX, the ras-mitogen-activated protein kinase pathway by a MEK inhibitor, and the rho pathway by C3 exoenzyme all substantially decreased nuclear signals from Edg receptors in both types of BCCs (Table 2).

Enhancement of BCC Secretion of IGF-II by LPA and S1P. Functional prominence of the IGF-II/IGFR1 system in many breast cancers suggested the possibility that part of the stimulation of proliferation of some lines of BCCs by LPA and/or S1P might be attributable to augmentation of secretion of IGF-II by one or both lysophospholipid mediators. Secretion of radioimmunoreactive IGF-II by MCF-7 BCCs was enhanced significantly by 10^{-9} M to 10^{-6} M LPA and S1P in concentration-dependent relationships where the maximal effects were attained by 10^{-6} M LPA and 10^{-8} M and 10^{-7} M S1P (Fig. 5). In two of the studies, neither 10^{-10} M LPA nor S1P affected release of IGF-II. At 10^{-6} M, but not 10^{-8} M, the phosphatidic acid and sphingosine biochemical precursors of LPA and S1P enhanced secretion of IGF-II with marginal statistical significance. A dot-blot immunoassay for IGF-II, which eliminates the blocking activity of IGF-binding proteins, gave similar results for MCF-7 BCCs. With 10^{-7} M LPA, 10^{-6} M LPA, 10^{-8} M S1P, and 10^{-7} M S1P, MCF-7 BCC-derived IGF-II was increased to respective means of 2.6-, 3.2-, 4.7-, and 5.5-fold above a mean unstimulated level of 2.2 ng/ml. Stimulation of MCF-7 BCC secretion of IGF-II by LPA and S1P was inhibited by PTX, MEK inhibition, and C3 exoenzyme sufficiently to implicate Gi and both the ras and rho pathways of signaling by the Edg receptors (Table 3). A greater involvement of signaling through the ras-raf-mitogen-activated protein kinase pathway than rho pathways may be predicted based on the higher effectiveness of the MEK inhibitor than C3 exoenzyme.

The level of secretion of IGF-II by LPA- and S1P-stimulated MDA-MB-453 BCCs was much lower than that by MCF-7 cells, and it was not possible to quantify accurately the very low IGF-II concentrations attained by unstimulated MDA-MB-453 cells. With 10^{-6} M LPA and 10^{-7} M S1P, the levels of IGF-II secreted by MDA-MB-453 BCCs attained means of 1.2 and 2.0 ng/ml, respectively. Because stimulated levels of IGF-II from MDA-MB-453 BCCs were only one-fifth of those from MCF-7 BCCs or lower and unstimulated levels were not reliably detectable, subsequent studies focused only on IGF-II mechanisms in MCF-7 BCCs. The capacity of human synthetic IGF-II to stimulate BCC proliferation, at concentrations in the range attained by incubation of MCF-7 BCCs with LPA and S1P, was examined to assess functional relevance of the observed endogenous increases. IGF-II increased MCF-7 BCC proliferation significantly, as determined by increases in cell counts after 72 h. MCF-7 BCC counts were increased by 1, 3, 10, and 30 ng/ml of IGF-II to respective means of 152, 234, 316, and 388% ($n = 2$) of serum-free medium control. The same range of concentrations of synthetic IGF-II also activated SRE in MCF-7 BCCs, as detected in the reporter assay (Table 4). The increases in SRE signal above control level were significant for all concentrations of IGF-II examined, and the increment in SRE signal attained by each higher concentration compared with the next lower concentration also was significant. The reduction in LPA-induced SRE signal by immunoneutralization of IGF-II was similar in magnitude to the maximum increase elicited by IGF-II alone (Table 4).

Suppression of MCF-7 BCC Responses to LPA and S1P by Anti-IGF-II and Anti-IGFR1 Antibodies. MCF-7 BCCs were preincubated with a range of concentrations of an IgG1 mouse neutralizing monoclonal anti-IGF II antibody, prior to introduction of 10^{-7} M LPA and S1P. The neutralizing antibody to IGF-II suppressed significantly both proliferative responses and SRE-luciferase reporter responses with antibody concentration dependence, whereas isotype-matched control IgG1 had no effect (Fig. 6). The effects of anti-IGFR1 antibody, which blocks binding of IGF-II to IGFR1, were examined in relation to the stimulatory effects of 10^{-7} M S1P on MCF-7 BCCs. At 1, 3, and 10 μg/ml, anti-IGFR1 antibody suppressed S1P-stimulated proliferation of MCF-7 BCCs, as assessed with cell counts, by means ± SD ($n = 3$) of 20 ± 4.6%, 32 ± 4.0%, and 41 ± 3.6% ($P < 0.01$ for all), respectively. At 3, 10, and 30 μg/ml, anti-IGFR1 antibody suppressed S1P-stimulated activation of the SRE-luciferase reporter in MCF-7 BCCs by means ± SD ($n = 3$) of 36 ± 7.8%, 47 ± 7.8%, and 51 ± 7.6% ($P < 0.01$ for all), respectively. In contrast, the IgG isotype control had no significant inhibitory effect, and anti-IGFR1 antibody did not suppress unstimulated proliferation of MCF-7 BCCs.

Table 3 *Pharmacological inhibition of LPA and S1P enhancement of MCF-7 BCC secretion of IGF-II*

Each value is the mean ± SD of the results of three studies. The significance of each level of inhibition was calculated by a paired Student t test. The levels of IGF-II in medium without an inhibitor were 7.4, 10, and 12 ng/ml for 10^{-7} M LPA and 6.1, 8.5, and 10 ng/ml for 10^{-7} M S1P.

	Lysophospholipid Signaling Inhibitor		
	PTX	MEK INH (mean inhibition ± SD)	C3 exoenzyme
LPA (10^{-7} M)	83 ± 14[a]	44 ± 8.3[a]	19 ± 15
S1P (10^{-7} M)	60 ± 19[b]	35 ± 3[a]	19 ± 2[b]

[a] $P < 0.01$.
[b] $P < 0.05$.

Table 4 *Activation of SRE-Luciferase reporter in MCF-7 BCCs by IGF-II*

Each value is the mean ± SD of the results of three studies. The significance of each level of stimulation relative to serum-free control without IGF-II or LPA (100%) was calculated by a paired Student *t* test. The levels of significance of differences between 1 and 3 ng/ml ($P < 0.01$), 3 and 10 ng/ml ($P < 0.05$), and 10 and 30 ng/ml ($P < 0.05$) of IGF-II and between LPA without and with anti-IGF-II neutralizing antibody ($P < 0.01$) were calculated by the same method.

IGF-II (ng/ml)					
1	3	10	30	LPA (10^{-7} M)	LPA (10^{-7} M) + anti-IGF-II (30 μg/ml)
178 ± 17[a]	209 ± 24[a]	260 ± 44[b]	316 ± 31[a]	1202 ± 152[a]	910 ± 165[a]

[a] $P < 0.01$.
[b] $P < 0.05$.

DISCUSSION

IGF-I and IGF-II potently stimulate proliferation of many types of normal and malignant cells (19, 20). The IGFR1 is a heterotetrameric complex with tyrosine kinase activity that binds and transduces signals from IGF-I and IGF-II similarly (21). IGFR2 differs structurally from IGFR1, lacks signal transduction functions, and does not mediate cellular proliferation (22). IGF-II is the predominant form in human cultured BCCs, stimulates BCC proliferation through IGFR1, and decreases the estrogen growth requirement of ER-positive BCCs (23). Estrogen is a potent stimulus of proliferation of ER-positive BCCs that concurrently enhances expression and secretion of IGF-II by such lines of BCCs (19). However, the possibility that the IGF system may not have a major role in estrogen enhancement of proliferation of some ER-positive BCCs was suggested by the lack of inhibition of estrogen stimulation when IGFR1 was blocked by a neutralizing monoclonal antibody (24). In contrast, stimulation of proliferation of BCCs by the lysolipid phosphate growth factors LPA and S1P appears to be mediated in part by IGF-II but is not dependent on the expression of ERs.

The ER-positive MCF-7 cells and ER-negative MDA-MB-453 cells both express Edg-2 and Edg-4 Rs for LPA and Edg-3 and Edg-5 Rs, but not Edg-1 Rs, for S1P, with quantitative differences in the respective levels (Figs. 1 and 2; Table 1). Significant ligand concentration-dependent stimulation of BCC proliferation by LPA and S1P was observed with both lines, irrespective of ER status (Fig. 3). Signaling of transcription of growth-related genes, as assessed by prominent enhancement of SRE-coupled luciferase activity, was increased significantly by proliferation-stimulating concentrations of LPA and S1P in both MCF-7 and MDA-MB-453 BCCs (Fig. 4). The suppression of SRE-coupled reporter responses to LPA and S1P by PTX and by inhibition of MEK and rho, in a pattern characteristic of signal transduction by Edg Rs, confirms the presence of functional Edg Rs in both BCC lines (Table 2).

LPA and S1P both significantly enhanced secretion of immunoreactive IGF-II by MCF-7 cells up to respective peaks 4- and 5-fold higher than control levels (Fig. 5). IGF-II secretion evoked by 10^{-7} M LPA or S1P was suppressed significantly by PTX and MEK inhibition and less significantly by C3 exoenzyme inactivation of rho, which also is consistent with Edg R mediation (Table 3). The role of IGF-II was explored first by investigating the stimulation of proliferation and SRE-luciferase activity in MCF-7 BCCs by a range of concentrations of purified synthetic IGF-II (Table 4). At concentrations elicited by LPA or S1P, the synthetic IGF-II evoked greater proliferation and SRE-luciferase activity than at concentrations attained by unstimulated MCF-7 BCCs. The role of native IGF-II was confirmed by defining the effects of neutralizing antibodies to IGF-II and IGFR1 on growth and SRE-reporter responses to 10^{-7} M LPA and S1P (Fig. 6). Both responses of MCF-7 cells were inhibited by means of up to 55 and 65%, respectively, without an effect of non-antibody isotype-identical IgG (Fig. 6). Thus, a substantial part of the stimulation of growth of some BCCs by LPA and S1P depends on increased release of IGF-II and its capacity to induce BCC proliferation.

A tentative integration of the present findings suggests distinctive functions for lysolipid phosphate mediators in BCC biology. At concentrations usually attained in serum and in some inflammatory and malignant exudates and plasma (1, 25, 26), LPA and S1P both exert dual effects on BCC proliferation. First, the SRE-luciferase responses not inhibited by anti-IGF-II or anti-IGFR1 neutralizing antibodies represent either direct nuclear signaling through Edg Rs or possibly the actions of other non-IGF protein growth factors elicited by the lysolipid phosphate mediators and capable of activating SRE. Second, LPA and S1P enhance generation and/or release of IGF-II by the

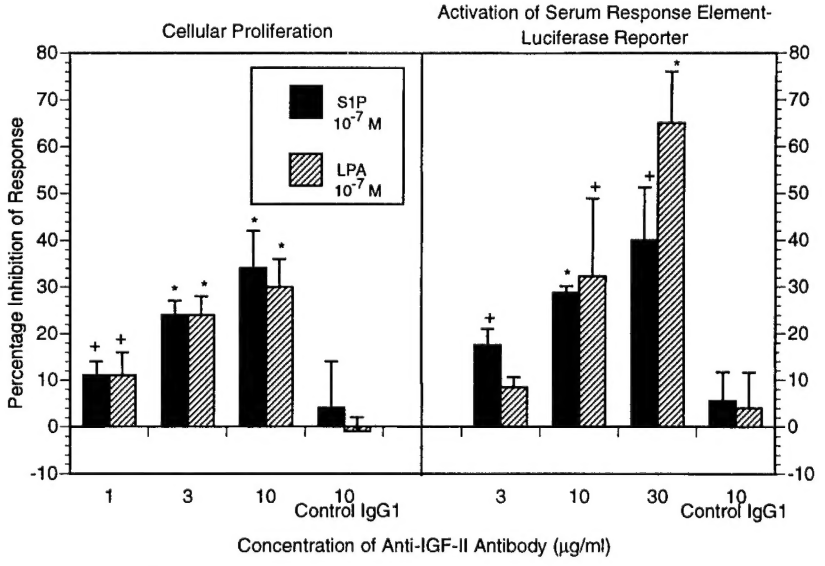

Fig. 6. Suppression of MCF-7 BCC responses to LPA and S1P by a neutralizing anti-IGF-II mouse monoclonal antibody. *Columns*, means of the results of three studies performed in duplicate; bars, SD. The control (0% inhibition) responses to 10^{-7} M LPA and S1P are shown in Figs. 3 and 4. The statistical methods and symbols are the same as in Fig. 4.

BCCs, irrespective of ER expression. The results of preliminary analyses of LPA and S1P production by BCCs showed very low endogenous levels, which would not have functional relevance. The sources of LPA and S1P, therefore, are likely to be cells other than the target BCCs, and these lysolipid phosphate growth factors thus would not appear to be autocrine stimuli in breast cancer. Rather, this class of mediators may function both as paracrine growth factors and by setting thresholds for secretory responses of one or more autocrine protein growth factors.

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REFERENCES

1. Fourcade, O., Simon, M. F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. Cell, *80:* 919–927, 1995.
2. Spiegel, S., and Milstein, S. Sphingolipid metabolites: members of a new class of lipid second messengers. J. Membr. Biol., *146:* 225–233, 1995.
3. Durieux, M. E., and Lynch, K. R. Signaling properties of lysophosphatidic acid. Trends Pharmacol. Sci., *14:* 249–254, 1993.
4. Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. Lysophosphatidic acid: G protein signaling and cellular responses. Curr. Opin. Cell Biol., *9:* 168–173, 1997.
5. Goetzl, E. J., and An, S. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. FASEB J., *12:* 1589–1598, 1998.
6. Hecht, J. H., Weiner, J. A., Post, S. R., and Chun, J. *Ventricular zone gene-1 (vzg-1)* encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. J. Cell Biol., *135:* 1071–1083, 1996.
7. An, S., Dickens, M. A., Bleu, T., Hallmark, O. G., and Goetzl, E. J. Molecular cloning of the human Edg-2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. Biochem. Biophys. Res. Commun., *231:* 619–622, 1997.
8. An, S., Bleu, T., Huang, W., Hallmark, O. G., Coughlin, S. R., and Goetzl, E. J. Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. FEBS Lett., *417:* 279–282, 1997.
9. An, S., Bleu, T., Hallmark, O. G., and Goetzl, E. J. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. J. Biol. Chem., *273:* 7906–7910, 1998.
10. Lee, M-J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S., and Hla, T. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG- 1. Science (Washington DC), *279:* 1552–1555, 1998.
11. Cuvillier, O., Rosenthal, D. S., Smulson, M. E., and Spiegel, S. Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. J. Biol. Chem., *273:* 2910–2917, 1998.
12. Goetzl, E. J., Kong, Y., and Mei, B. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. J. Immunol., *162:* 2049–2056, 1999.
13. Hill, C. S., and Treisman, R. Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. EMBO J., *14:* 5037–5042, 1995.
14. An, S., Bleu, T., Zheng, Y., and Goetzl, E. J. Recombinant human Edg-2 and Edg-4 lysophosphatidic acid receptors mediate intracellular calcium mobilization. Mol. Pharmacol., *54:* 881–888, 1998.
15. Goetzl, E. J., Kong, Y., and Kenney, J. S. Lysophospholipid enhancement of human T cell sensitivity to diphtheria toxin by increased expression of heparin-binding epidermal growth factor. Proc. Assoc. Am. Physic., *111:* 1–11, 1999.
16. Kaltreider, H. B., Ichikawa, S., Byrd, P. K., Ingram, D. A., Kishiyama, J. L., Sreedharan, S. P., Warnock, M. L., Beck, J. M., and Goetzl, E. J. Upregulation of neuropeptides and neuropeptide receptors in a murine model of immune inflammation in lung parenchyma. Am. J. Resp. Cell. Mol. Biol., *16:* 133–145, 1996.
17. Crawford, B. A., Martin, J. L., Howe, C. J., Handelsman, D. J., and Baxter, R. C. Comparison of extraction methods for insulin-like growth factor-I in rat serum. J. Endocrinol., *134:* 169–176, 1992.
18. DeLeon, D. D., Terry, C., and Nissley, S. P. Direct detection of insulin like growth factor II (IGF-II) by chemiluminescence without interference by IGF binding proteins. Endocrinology, *134:* 1960–1963, 1994.
19. Stewart, A. J., Johnson, M. D., May, F. E. B., and Westley, B. R. Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells. J. Biol. Chem., *265:* 21172–21178, 1994.
20. Yee, D. The insulin-like growth factor system as a target in breast cancer. Breast Cancer Res. Treat., *32:* 85–95, 1994.
21. Steele-Perkins, G., Turner, J., Edman, J. C., Hari, J., Pierce, S. B., Stover, C., Rutter, W. J., and Roth, R. A. Expression and characterization of a functional human IGF-I receptor. J. Biol. Chem., *263:* 11486–11492, 1988.
22. Roth, R. A. Structure of the receptor for IGF-II: the puzzle amplified. Science (Washington DC), *239:* 1269–1271, 1988.
23. Daly, R. J., Harris, W. H., Wang, D. Y., and Darbre, P. D. Autocrine production of insulin-like growth factor II using an inducible expression system results in reduced estrogen sensitivity of MCF-7 human breast cancer cells. Cell Growth Differ., *2:* 457–464, 1991.
24. Arteaga, C. L., and Osborne, C. K. Growth inhibition of human breast cancer cells *in vitro* with an antibody against the type I somatomedin receptor. Cancer Res., *49:* 6237–6241, 1989.
25. Xu, Y., Gaudette, D. C., Boynton, J., and Mills, G. B. Characterization of an ovarian cancer activating factor (OCAF) in ascites from ovarian cancer patients. Clin. Cancer Res., *1:* 1223–1232, 1995.
26. Xu, Y., Shen, Z., Wiper, D. W., Wu, M., Morton, R. E., Elson, P., Kennedy, A. W., Belinson, J., Markman, M., and Casey, G. Lysophosphatidic acid as a potential biomarker in ovarian and other gynecological cancers. J. Am. Med. Assoc., *280:* 719–723, 1998.